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FULL PAPER

Evaluation of a combination of alfaxalone with medetomidine and butorphanol for inducing surgical anesthesia in laboratory mice

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Abstract

The anesthetic effects of alfaxalone were investigated in mice. Mice were administered alfaxalone (100 mg/kg) alone or the combinations of 0.3 mg/kg of medetomidine and 5 mg/kg of butorphanol with alfaxalone at doses of 20 mg/kg (M/B/A20), 40 mg/kg (M/B/A40), 60 mg/kg (M/B/A60), or 80 mg/kg (M/B/A80). Control mice received 0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol (M/M/B). Each drug was administrated by intraperitoneal (IP) or subcutaneous (SC) routes. M/M/B IP did not achieve surgical anesthesia but M/M/B SC achieved surgical anesthesia within 10 min after administration and maintained anesthesia for 45 min. The anesthetic scores were very low after IP or SC administration of alfaxalone alone. M/B/A20 IP and SC did not achieve surgical anesthesia. M/B/A40 IP did not achieve surgical anesthesia but M/B/A40 SC achieved surgical anesthesia within 10 min after administration and maintained anesthesia for 35 min. M/B/A60 SC achieved surgical anesthesia within 5 min after administration and maintained anesthesia for 75 min. By contrast, M/B/A60 IP did not achieve surgical anesthesia. M/B/A80 SC achieved surgical anesthesia within 5 min after administration and maintained anesthesia for 85 min. By contrast, M/B/A80 IP did not achieve surgical anesthesia and one mouse died about 10 min after drug administration. Administration of atipamezole rapidly reversed anesthesia induced by M/B/A60 in mice. These results suggest that M/B/A60 SC, an alfaxalone-based combination, is suitable for inducing surgical anesthesia in laboratory mice.

Key Words: alfaxalone, anesthesia, butorphanol, medetomidine, mice

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Introduction

In inducing anesthesia in laboratory mice for the purpose of biomedical research, anesthetics are usually delivered via the intraperitoneal (IP) route. After ketamine was classified as a narcotic drug in Japan in 2007, several combinations of sedative and analgesic drugs, especially medetomidine, midazolam, and butorphanol, have been used to induce anesthesia in laboratory rodents[12,14]. Generally, mice are injected via the IP route with combination of 0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol[12,14]. However, it has been reported that the doses of these drugs in this combination are not used consistently between different research groups[9,16]. Furthermore, the injection route used may affect the duration of anesthesia[15]. It is also notable that this combination comprises three sedative and analgesic drugs, but not an anesthetic drug.

Alfaxalone (3α-hydroxy-5α-pregnane-11,20-dione) is an injectable neurosteroid anesthetic[33] that modulates the γ-aminobutyric acid A ($GABA_A$) receptor and causes neurodepression and muscular relaxation[1,11,17]. Because of its water insolubility, previous alfaxalone products (e.g., Althesin®, Saffan®) were solubilized in 20% polyoxyethylated castor oil (Cremophor EL) and coformulated with a related neurosteroid, alfadolone. However, this product was voluntary withdrawn from the market because of its side effects induced by histamine release associated with the solubilizing agent[2,6]. Recently, alfaxalone was reformulated with another solubilizing agent, 2-hydroxypropyl-β-cyclodextrin, which does not cause histamine release, and the new formulation is registered for use in dogs and cats as Alfaxan® (Jurox Pty Ltd., Rutherford, NSW, Australia; hereafter alfaxalone). This formulation is now widely used to induce anesthesia and provides satisfactory induction of anesthesia in dogs[7,24,30] and cats[25,34]. Alfaxalone has few or no cardiovascular effects when given at clinical doses, unlike propofol[13,22,26]. In order to improve the safety and quality of anesthetic induction, several studies have tested combinations of alfaxalone with sedatives and opioids in dogs[19,27,29]. However, few studies have evaluated the effects of alfaxalone combined with sedatives and opioids in small laboratory animals. Therefore, this study was performed to investigate the anesthetic effects of alfaxalone combined with medetomidine and butorphanol in laboratory mice.

Materials and Methods

This study was carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan (http://www.scj.go.jp/en/animal/index.html). All animal procedures and their care were approved by the Animal Care and Use Committee of Rakuno-Gakuen University in accordance with the Guide for the Care and Use of Laboratory Animals.

Mice: Specific pathogen-free female ICR mice, aged 5–6 weeks, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were housed in autoclaved polycarbonate cages with autoclaved bedding under barrier-sustained conditions and controlled temperature (23 ± 2°C) and lighting (12-h light/dark cycle). Mice were fed a commercial diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and received tap water *ad libitum*. Mice were allowed to acclimatize for at least 1 week before use at 7 weeks of age (body weight 28.3 ± 2.0 g).

Combination of anesthetic drugs: The following anesthetic drugs were used in this study: medetomidine (Domitol®; Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), midazolam (Dormicum®; Astellas Pharma Inc., Tokyo, Japan), butorphanol (Vetorphale®; Meiji Seika Pharma Co., Ltd.), and alfaxalone. A medetomidine antagonist, atipamezole (Antisedan®, Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), was used to reverse

Table 1. Summary of the drugs and doses used in this study

Abbreviation	Dose (mg/kg)				Injection route[1)]	No. of mice
	Medetomidine	Midazolam	Butorphanol	Alfaxalone		
IP M/M/B	0.3	4	5	–	IP	5
IP ALFX	–	–	–	100	IP	5
IP M/B/A20	0.3	–	5	20	IP	5
IP M/B/A40	0.3	–	5	40	IP	5
IP M/B/A60	0.3	–	5	60	IP	6
IP M/B/A80	0.3	–	5	80	IP	5
SC M/M/B	0.3	4	5	–	SC	5
SC ALFX	–	–	–	100	SC	5
SC M/B/A20	0.3	–	5	20	SC	5
SC M/B/A40	0.3	–	5	40	SC	5
SC M/B/A60	0.3	–	5	60	SC	6
SC M/B/A80	0.3	–	5	80	SC	5

[1)]IP, intraperitoneal; SC, subcutaneous.

anesthesia in some experiments. The combinations of anesthetic drugs that were used are listed in Table 1. Drugs were diluted in normal saline (0.9% NaCl) to a concentration that could be administered in a total volume of 0.01 ml/g of body weight. M/M/B comprised 0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol. Other groups of mice were administered alfaxalone alone at a dose of 100 mg/kg or the combinations of 0.3 mg/kg of medetomidine and 5 mg/kg of butorphanol with alfaxalone at doses of 20 mg/kg (M/B/A20), 40 mg/kg (M/B/A40), 60 mg/kg (M/B/A60), or 80 mg/kg (M/B/A80).

Experimental protocols: Mice were randomly allocated to 12 groups, and each group received one anesthetic protocol (summarized in Table 1).

Each group contained ≥ 5 mice, and groups were administered the drugs via IP or subcutaneous (SC) routes. After drug injection, the mouse was kept on a heater plate (FHP450-S; Tokyo Glass Kikai, Co., Ltd., Tokyo, Japan) maintained at approximately 38°C. The reflex response to a stimulating noxious stimulus was tested every 5 min for 90 min, and at 120, 150, and 180 min after drug administration. To investigate whether administration of atipamezole was effective in reversing anesthesia induced by M/B/A60 SC, 10 mice received M/B/A60 SC, which was followed 60 min later by SC administration of 0.3 mg/kg of atipamezole (n = 5) or normal saline (n = 5).

Assessment of anesthetic depth: The reflex response to a stimulus was assessed using a method reported by Kawai *et al.* with some modifications[12)]. Five reflexes were evaluated: the righting reflex, the fore- and hind-limb pedal withdrawal reflexes, the tail pinch reflex, and the eyelid reflex. The righting reflex was assessed by placing the mouse on its back and observing the motion taken to correct its posture to determine the presence (score = 0) or absence (score = 1) of a reflex. The tail pinch reflex was assessed in six locations by pinching the proximal tail lightly with atraumatic forceps, and observing the presence (score = 0) or absence (score = 1) of a reflex. The pedal withdrawal reflex was assessed by lightly pinching the interdigital webbing of all four limbs using atraumatic forceps, and observing the presence (score = 0) or absence (score = 1) of a reflex for the fore- and hind-limbs. The eye reflex was assessed by blowing air onto the cornea using a Pasteur pipette with a 2 ml silicone nipple, and observing the presence (score = 0) or

absence (score = 1) of a reflex. Each parameter was scored, and the anesthetic depth was expressed as the total score for each mouse. A score of ≥ 4 was defined as surgical anesthesia. The time to the loss of the righting reflex and the time to recovery of the righting reflex were also recorded. The immobilization time (i.e., the time during which the animal made no movements) was defined as the time from the loss of the righting reflex and the recovery of the righting reflex. All tests were performed by investigators who were not blinded to the study treatments.

Statistical analysis: All data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical comparisons among three or more groups and Student's *t* test was used for comparisons between pairs of groups. Values of $P < 0.05$ were considered statistically significant. The free statistical software R (version 3.2.2) was used for all analyses (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Comparison of anesthetic scores between combinations of anesthetic drugs

The highest mean score for the most commonly used anesthetic combination of 0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol (M/M/B) administered IP was 3.6 at 50 min after M/M/B injection. M/M/B did not achieve a mean score of 4, defined as surgical anesthesia, at any time (Fig. 1a). Furthermore, an anesthetic effect was not observed in one of five mice in this group. However, M/M/B achieved surgical anesthesia at 10 min after SC administration, and anesthesia was maintained for 45 min (Fig. 1a). The immobilization times following IP and SC administration were 48.2 ± 27.2 min and 70.6 ± 3.3 min, respectively (Fig. 2).

In the 100 mg/kg alfaxalone group, the anesthetic scores were very low, consistently < 2, following IP and SC administration (Fig. 1b). The immobilization times following IP and SC administration were 40.4 ± 2.9 min and 60.0 ± 5.0 min ($P < 0.05$ vs. IP), respectively (Fig. 2).

In the alfaxalone combination groups, M/B/A20 did not achieve surgical anesthesia by either route (Fig. 1c), and an anesthetic effect was not observed in two of five mice in the M/B/A20 IP group. The immobilization times following IP and SC administration of M/B/A20 were 12.4 ± 5.0 min and 34.6 ± 0.8 min ($P < 0.05$ vs. IP), respectively (Fig. 2).

M/B/A40 IP did not achieve surgical anesthesia, but M/B/A40 SC achieved surgical anesthesia by 10 min after drug administration, and anesthesia was maintained for 35 min (Fig. 1d). M/B/A40 IP did not have an anesthetic effect in three of five mice. The immobilization times following IP and SC administration were 15.8 ± 9.8 min and 69.8 ± 2.8 min ($P < 0.05$ vs. IP), respectively (Fig. 2).

M/B/A60 SC achieved surgical anesthesia by 5 min after administration, and anesthesia was maintained for 75 min. By contrast, M/B/A60 IP did not achieve surgical anesthesia (Fig. 1e). An anesthetic effect was not observed in four mice in the M/B/A60 IP group. The immobilization times following IP and SC administration were 26.0 ± 18.1 min and 97.0 ± 3.2 min ($P < 0.05$ vs. IP), respectively (Fig. 2).

M/B/A80 SC achieved surgical anesthesia by 5 min after administration, and anesthesia was maintained for 85 min. By contrast, M/B/A80 IP did not achieve surgical anesthesia (Fig. 1f). One mouse in the IP group died about 10 min after drug administration; this mouse was excluded from Fig. 1f. In addition, an anesthetic effect was not observed in one mouse in this group. The immobilization times following IP and SC administration were 82.0 ± 33.6 min and 118.0 ± 5.2 min, respectively. There were no significant differences between IP and SC administration.

There were no significant complications or

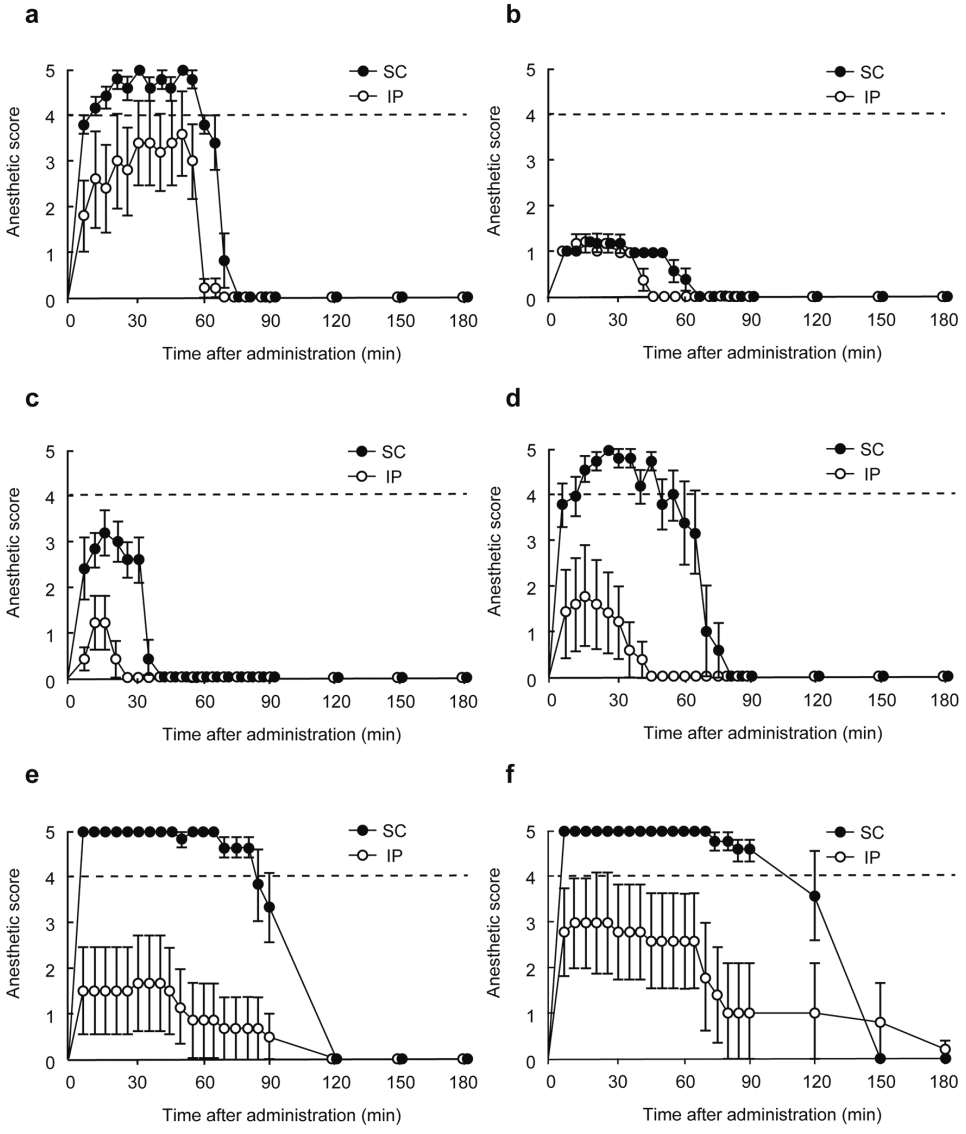

Fig. 1. Anesthetic scores following subcutaneous and intraperitoneal administration of each combination of anesthetic drugs. (a) 0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol (M/M/B). (b) 100 mg/kg of alfaxalone (ALFX) alone. (c) 0.3 mg/kg of medetomidine, 5 mg/kg of butorphanol, and 20 mg/kg of alfaxalone (M/B/A20). (d) 0.3 mg/kg of medetomidine, 5 mg/kg of butorphanol, and 40 mg/kg of alfaxalone (M/B/A40). (e) 0.3 mg/kg of medetomidine, 5 mg/kg of butorphanol, and 60 mg/kg of alfaxalone (M/B/A60). (f) 0.3 mg/kg of medetomidine, 5 mg/kg of butorphanol, and 80 mg/kg of alfaxalone (M/B/A80). Closed circles, subcutaneous administration; open circles, intraperitoneal administration. Surgical anesthesia was defined as a total anesthetic score of > 4 (dashed line). Results are presented as the mean $\pm$ SEM. IP: intraperitoneal; SC: subcutaneous.

side effects of the anesthetic drugs in any of the groups, except for one death in the M/B/A80 IP group.

Differences in anesthetic scores between the drug combinations and administration route

Fig. 3 compares the effects of IP and SC administration of each combination. None of the IP combinations achieved surgical anesthesia in this study (Fig. 3a). Compared with M/M/B IP, only M/B/A80 IP was associated with higher anesthetic scores at 60 and 65 min after administration ($P < 0.05$). Following SC administration, the combinations M/M/B SC, M/B/A40 SC, M/B/A60

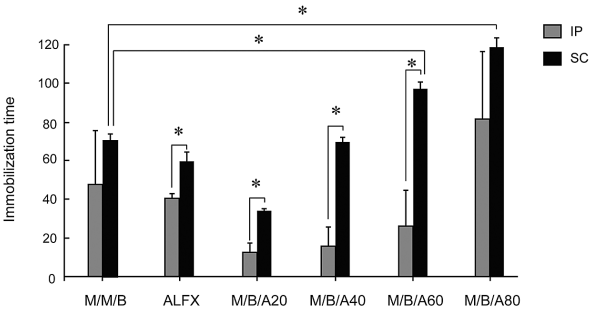

Fig. 2. Comparison of immobilization times after subcutaneous and intraperitoneal administration of each combination of drugs. The immobilization time was defined as the time from the loss of the righting reflex to the recovery of the righting reflex. Results are presented as the mean ± SEM. One-way ANOVA analysis of variance followed by Dunnett's test was used for comparisons between M/M/B (as a control) and the other groups by IP or SC administration. Student's *t* test was used to compare the effects of the IP and SC routes. $*P < 0.05$. ALFX: alfaxalone. Other abbreviations are defined in Table 1 and Fig. 1.

SC, and M/B/A80 SC achieved surgical anesthesia, which was maintained for 45, 35, 75, and 85 min, respectively (Fig. 3b). Compared with M/M/B SC, the anesthetic scores for M/B/A60 SC were higher at 5, 10, 15, 60, 65, 70, 75, 80, 85, and 90 min after administration, while the scores for M/B/A80 SC were higher at 5, 10, 15, 60, 65, 70, 75, 80, 85, 90, and 120 min after administration ($P < 0.05$). The immobilization times for both M/B/A60 SC and M/B/A80 SC were significantly greater than that of the standard combination M/M/B SC ($P < 0.05$). There was little variability in the immobilization times for each SC combination among the individual mice, as compared with those for the IP combinations (Fig. 2).

Antagonistic effects of atipamezole after administration of M/B/A60

Mice were SC administered with atipamezole at 60 min after SC administration of M/B/A60. The mice recovered quickly, within 15 min, after administration of atipamezole (Fig. 4). The anesthetic scores of mice treated with atipamezole was significantly decreased compared with that of normal saline injection from 5 min after injection.

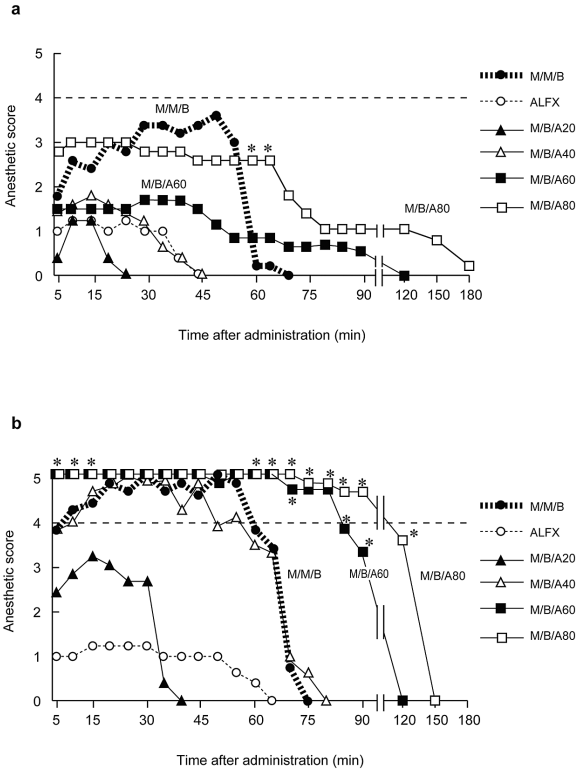

Fig. 3. Comparison of anesthetic scores over time after subcutaneous and intraperitoneal administration of each combination of drugs. (a) Intraperitoneal administration. (b) Subcutaneous administration. Surgical anesthesia was defined as a total anesthetic score of > 4 (dashed line). Close circles, M/M/B; open circles, alfaxalone alone; closed triangles, M/B/A20; open triangles, M/B/A40; closed squares, M/B/A60; open squares, M/B/A80. $*P < 0.05$ compared with M/M/B group (Dunnett's test). ALFX: alfaxalone. Other abbreviations are defined in Table 1 and Fig. 1.

Discussion

The results of this study indicate that SC administration of a combination of medetomidine, butorphanol, and 60 mg/kg of alfaxalone (i.e. M/B/A60) was suitable for inducing anesthesia in laboratory mice, and was more effective than the other tested combinations. Administration of atipamezole[5,32], quickly reversed the anesthetic effects of M/B/A60.

Surgical anesthesia was achieved with the SC combinations M/B/A40, M/B/A60, M/B/A80, and M/M/B (as a control). The immobilization time and anesthetic scores for M/B/A40 SC were similar to those of M/M/B SC. By contrast, M/B/

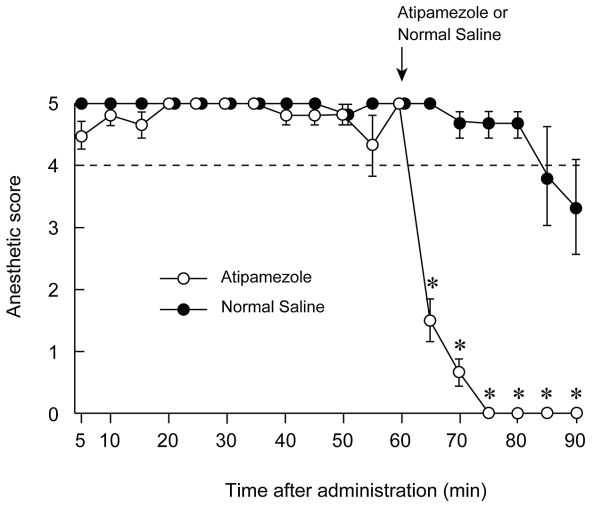

Fig. 4. Antagonistic effects of atipamezole in mice administered with M/B/A60. Atipamezole (0.3 mg/kg) was administered subcutaneously 60 min after subcutaneous administration of M/B/A60. Surgical anesthesia was defined as a total anesthetic score of >4 (dashed line). Results are presented as the mean $\pm$ SEM (n = 5 per group). *$P < 0.05$ (Student's t test).

A60 SC and M/B/A80 SC were associated with significantly greater immobilization times and anesthetic scores compared with M/M/B SC. In addition, M/B/A60 SC and M/B/A80 SC achieved surgical anesthesia at 5 min after administration. However, one mouse administered with M/B/A80 IP died; therefore, we think we should avoid SC administration of 80 mg/kg of alfaxalone in combination with other anesthetic drugs. This study revealed that M/B/A had anesthetic effects in mice, and these effects increased with increasing dose of alfaxalone. Although apnea, muscle twitching and myoclonic jerk were reported as side effects of alfaxalone in rats and mice[8,18], these were not observed in this study.

Although IP administration of drugs is widely believed to be more effective for inducing anesthesia in laboratory animals, including mice[10,31], SC administration has several advantages, including reduced injury, stress, and partial failure rates, compared with IP administration[21]. For M/M/B, Kirihara *et al.*[15] reported that SC administration shows a tendency to prolong anesthesia compared with IP administration, although the difference was not statistically significant. In the current study, we also found that SC administration of M/M/B achieved better anesthetic scores and longer immobilization time compared with IP administration, although the differences were not statistically significant. It was reported that IP administration fails to achieve anesthesia in 10%–20% of animals[4]. IP injection involves insertion of the needle through the abdominal wall into the peritoneal cavity, and there is no visual confirmation that the injection has been correctly performed. By contrast, it is much easier to observe administration failure using the SC route[10]. Nevertheless, because the results reported by Kirihara *et al.*[15] and our group were consistent, the risk of technical failure is low. Surprisingly, we observed significant differences between IP and SC administration of alfaxalone alone, M/B/A20, M/B/A40, and M/B/A60. For each of these combinations, the immobilization time was longer with SC administration than with IP administration. Lau *et al.*[18] reported that immobilization was not achieved in 30% of rats following IP administration of alfaxalone, possibly because of the lower initial plasma concentrations of alfaxalone in these animals. These results suggest that the limited effect of IP administration of alfaxalone might be due to a high first-pass elimination of alfaxalone via hepatic metabolism[3,23,28], because IP administered drugs predominantly enter the portal circulation[20]. Further studies are needed to fully explain the mechanism underlying the significant differences in anesthetic duration between IP and SC administration of alfaxalone-based combinations of anesthetics.

To our knowledge, this was the first study to examine the anesthetic properties of alfaxalone alone or in combination with other commonly used drugs, as well as the effects of the anesthetic route on the induction and duration of anesthesia. Based on our results, we recommend the combination of SC M/B/A60 as a reliable anesthetic drug for murine surgery.

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Conflict of interest statement

The authors declare no conflicts of interest.

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